conformational changes that occur on ligand binding with systematic energy based rotational methods and maps the allosteric conformational changes with MD simulations. Receptor conformational states for rhodopsin bound to trans-retinal, β2-adrenergic receptor bound to five different ligands with varied efficacies (agonist, partial agonists of two different chemical structure, weak partial agonist, inverse agonist). The inter-residue distances obtained from these predictions will be correlated to experimental results and discussed. The role of allosteric antagonists in inducing conformational changes in chemokine receptors CCR2, CCR5 and CCR3 will be discussed. I will also discuss on how the results from this study can be applied in designing mutation/fluorescence experiments to study activation mechanism and designing drugs that fit a particular receptor conformation.

2681-Plat Genetics, Nanotechnology And A New Microscope (PAM) Reveal Molecular Details Of ErbB Tyrosine Kinase Receptors

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/>Activation of the erbB receptors by the extracellular binding of peptide ligands triggers signaling cascades for cellular motility, cell division, and differentiation. We genetically tagged ErbB proteins with fluorescent proteins or acyl carrier protein (ACP) sequences. Photo- and chemically-stable semiconductor "quantum dots" (QDs), were targeted to receptors on the external cell surface. By combining these ligands and new, high-resolution microscopy techniques, we gain insights into molecular interactions and downstream signaling pathways. The results were acquired with a new generation, commercial, Programmable Array optically sectioning fluorescence Microscope (PAM) for rapid (20 Hz), light-efficient 3D imaging. The stand-alone module, including light source(s) and detector(s), features a ferroelectric liquid-crystal-on-silicon (LCoS) spatial light modulator.

(1) *Activated ErbB1 mobility and retrograde transport*. QD-coupled EGF allows visualization in living cells of individual EGFR receptors, the diffusion of which has been determined on different cell types with the PAM. Utilizing these probes we discovered a systematic retrograde transport on filopodia of EGFR following EGF binding and activation. The process is linked to treadmilling of actin filaments. This phenomenon acts as a biosensor, in that receptors are transferred from remote sites of activation to the transduction mechanisms in the cell body. We have mutated specific tyrosine residues in the cytoplasmic tail of the EGFR to identify the adaptor molecules mediating the transport as well as used peptides that cause a dominant negative phenotype in live cell PAM imaging.

(2) *Partition of activated receptor complexes*. The fate of activated receptors determines the extent and magnitude of signaling, and may provide insights as to how to inhibit oncogenic growth. Pulse chase experiments with the PAM shed new light on the effect of various ligands in cells expressing different combinations of the RTKs.

2682-Plat Imaging Avidity: T Cell Receptor Aggregation Measured in Live Cells using Quantum Dots and Image Correlation Spectroscopy

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Changes in receptor organization are used by both prokaryotic and eukaryotic cells as a means of modulating their dynamic ranges of responsiveness to environmental cues. Specifically within the immune system this has been studied when naïve T cells become activated after antigenic stimulation (Fahmy, et al., Immunity 2001; *14*:135–143). We present a novel method of measuring T cell receptor (TCR) aggregation in live cells using quantum dot labeling and image correlation spectroscopy. 2C TCR transgenic cells in culture were observed from the naïve state to 12 days after activation. Cells were first labeled with biotin-MHC/peptide known to bind the TCR, and then streptavidin-quantum dots before imaging on an emCCD-equipped microscope, giving single-dot imaging sensitivity. The image series acquired were analyzed using k-space image correlation spectroscopy (Kolin, et al., Biophys. J. 2006; *91*:3061–3075), which allowed the aggregation of TCR to be quantified by two different approaches. The first uses spatial intensity fluctuations in an image to measure the clustering of receptors, while the second detects activation via changes in the intensity correlation function of the blinking QDs. In contrast to fluorescence activated cell sorting, which requires ~10^5 cells, our new approach can detect the activation state on individual cells and only requires ~40 cells. T cells exhibited maximum activation 3–4 days after initial activation, when their TCR degree of aggregation was an order of magnitude greater than the naïve state. This new technology has powerful applications as it can be applied to just a few cells and could be used to detect microheterogenity in receptor organization of cells in vivo.

Platform BG: Self-Assembled Session: Calsequestrin and the Cellular Ca^{+} Store of Skeletal and Cardiac Muscle

2683-Plat Lumenal Calcium Regulation of Single Cardiac Ryanodine Receptor Channels

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The lumenal Ca^{2+} regulation of cardiac ryanodine receptor (RyR2) was explored at the single channel level. Heavy SR microsomes isolated from rat cardiac muscle were fused into planar bilayers. Two lumenal Ca^{2+} regulatory mechanism(s) were identified and distinguished by their Ca^{2+} vs. Mg^{2+} sensitivity. One is a RyR2-resident mechanism which is likely mediated by a lumenal Ca^{2+} site on the channel protein itself. This mechanism operates in the

absence of CSQ2, does not distinguish between lumenal Ca^{2+} and Mg^{2+}, and determines the maximal efficacy of large cytosolic Ca^{2+} stimuli. The other mechanism is CSQ2 dependent lumenal Ca^{2+} regulation. This mechanism does distinguish between lumenal Ca^{2+} and Mg^{2+}. It shifts the cytosolic Ca^{2+} affinity of the RyR2 channel. It does not seem to depend on CSQ2 oligomerization or CSQ2 monomer Ca^{2+} binding affinity. Its lumenal Ca^{2+} "sensing" appears to be associated with Ca^{2+}-dependent CSQ2 interaction with the unglycosylated form of Triadin. This mechanism was also explored by substituting wildtype (wt) for mutant CSQ2 (R33Q & L167H). R33Q CSQ2 participated in lumenal RyR2 Ca^{2+} regulation but less effectively than wt CSQ2. However, L167H CSQ2 did not participate in lumenal RyR2 Ca^{2+} regulation. The disparate actions of these two catecholaminergic polymorphic ventricular tachycardia (CPVT) linked mutants implies that alteration or elimination of CSQ2-dependent RyR2 regulation can generate the CPVT phenotype. We propose that the RyR2-resident lumenal Ca^{2+} mechanism may assure all channels (CSQ-free or bound) respond robustly to large local cytosolic Ca^{2+} stimuli (>5 μM) while the CSQ2-dependent mechanism helps close RyR2 channels (terminate release) after lumenal Ca^{2+} falls below ~0.5 mM. Disrupting/eliminating the later makes channels more likely to reopen until intra-SR Ca^{2+} levels return to normal values (~1 mM) and this generates the CPVT phenotype.

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2684-Plat Isoform dependent properties of CSQ

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CSQ is the major Ca^{2+} binding protein and luminal Ca^{2+} sensor in skeletal and cardiac muscle. However CSQ1 (skeletal) and CSQ2 (cardiac) are products of different genes and share only 66~80% homology in their primary sequence. We show that the Ca^{2+} binding capacity and associations with the RyR of the two CSQ isoforms are profoundly different. The Ca^{2+} binding capacity of rabbit skeletal muscle CSQ1 and sheep cardiac CSQ2 were determined using a ^{45}Ca binding assay and confirmed using StainsAll staining. Despite the fact that CSQ2 has an extended acidic C-terminal tail (~30 residues longer than CSQ1), CSQ2 binds half as much Ca^{2+} as CSQ1. The fraction of CSQ associated with the SR membrane was quantified following SDS-PAGE of SR fractions and Western blotting with antibodies against CSQ1 and CSQ2. The SR, prior to 0.5% Triton X-100 solubilization (using 1 mM Ca^{2+} to retain CSQ's polymer structure), was examined as well as the supernatant and membrane fraction after centrifugation of the solubilized material. We found that 80% of CSQ1, but only 20% of CSQ2, was membrane-associated. The ratio of CSQ to RyR in the SR was assessed from the solubilized membrane samples using Western Blot with anti-CSQ1 and anti-CSQ2 antibodies. The membrane samples contained equal amounts of RyR1 and RyR2 and the anti-CSQ antibodies used in concentrations determined to stain CSQ1 and CSQ2 with equal density. The CSQ/RyR ratio was ~4-fold greater in skeletal than in cardiac muscle. These results indicate that either CSQ is less polymerized in the heart, or less CSQ polymer is associated with triadin/junctin/RyR. The dissimilar properties of CSQ1 and CSQ2 suggest a differential regulation of their respective SR Ca^{2+} stores which may underlie the strong store depletion with each cardiac contraction, but retention of store Ca^{2+} load in skeletal muscle.

2685-Plat The Effects Of Severe Knock-down Of Calsequestrin 1 In Adult Mammalian Muscle

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Calsequestrin is the major calcium binding protein in the SR. Altering its concentration is expected to profoundly alter Ca^{2+} signaling. We transiently suppressed synthesis of CSQ1 in predominantly fast twitch muscle of live adult mice by injection into hind paws of a plasmid vector (pSilencer, Ambion) including code for siRNA tested by Wang et al. (2006). Of four injections, done at 5-day intervals, two used a bicistronic vector including EGFP code. Immunoblots showed reduction of CSQ1 greater than 95% in the whole treated FDB muscle compared with the contralateral mock-injected muscle. In the same muscles the level of CSQ2 (which is immunologically detectable in 20% of normal cells; Paolini et al. 2007) was not affected.

Whole muscle twitch and tetanic tension, force-frequency relationship and development of fatigue showed no changes of treated vs. controls (3 pairs). Ca transients and Ca release flux were measured in fibers enzymatically separated, selected by high expression of GFP and patch clamped, using a combination of SEER (Launikonis et al. 2005) of indo-1 and fluorescence of X-rhod. No significant changes were detected in total releasable Ca or flux kinetics. Challenges with 0.5–2 mM chloro-m-cresol found no significant reduction in releasable Ca in 9 silenced fibers (vs 6 mock-treated cells). Electron microscopy of silenced cells revealed largely intact triads but some terminal cisternae appeared dilated and had low density content. The virtual absence of functional effects associated with CSQ1 silencing or overexpression (Royer et al. this meeting) as regards calcium load, its movements and mechanical output at the organ level, is difficult to reconcile with prevailing notions on the roles of calsequestrin in skeletal muscle function.

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2686-Plat Calcium buffering properties of calsequestrin

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We recently evaluated the role of calsequestrin in frog cut skeletal muscle fibers by measuring the free and total Ca within the SR - [Ca^{2}

$^{+}]_{SR}$ and $[Ca_T]_{SR}$, respectively - in response to fully-depleting stimulations (J. Physiology, 2007, 581.1:318–367). $[Ca_T]_{SR}$ was measured with the EGTA-phenol red method. $[Ca^{2+}]_{SR}$ was assessed with the membrane-permeable absorbance indicator dye tetramethymurexide (TMX). Due to TMX's low affinity for Ca (K_D = 2.6 mM) and the presence of 20 mM EGTA in the myoplasm, Ca binding to TMX in the myoplasm was negligible so that essentially all of the CaTMX signal was from the SR. One difficulty involved uncertainty about an important parameter, the resting value of $[Ca^{2+}]_{SR}$ ($[Ca^{2+}]_{SR,R}$). In a study described elsewhere at this meeting, we estimated $[Ca^{2+}]_{SR,R}$ by permeabilizing the surface membrane of isolated fibers with saponin, thereby rapidly washing TMX from the myoplasm leaving TMX in the SR only. The value of $[Ca^{2+}]_{SR,R}$ was 0.7 mM, which is close to that estimated by indirect means in the above report. Combined with the report above, our results indicate that calsequestrin has a very high capacity for buffering Ca with 23 times more Ca coming from calsequestrin as opposed to the pool of free Ca^{2+}. Moreover, Ca binding is highly cooperative (Hill coefficient of ~3) with a K_D for Ca of ~1 mM. This means that calsequestrin is optimized for buffering Ca near the physiological value of $[Ca^{2+}]_{SR,R}$. Calculations suggest that the diffusion-limited (maximum) steady-state Ca^{2+} flux through an open SR Ca^{2+} release channel in the absence of calsequestrin could be significantly less than the actual flux. Therefore, an important function of calsequestrin may be to enable larger fluxes by preventing the local decrease in $[Ca^{2+}]_{SR}$ near the luminal side of the SR Ca^{2+} release channel.

2687-Plat Heat and Anesthesia cause a Lethal Crisis in Calsequestrin-1 Null Mice

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Our recently published results in mice lacking skeletal calsequestrin (Paolini et al. 2007, *J. Physiol.* 583:767) indicates that, in fast twitch fibers, CS1 is important for SR structure, Ca^{2+} storage and possibly to facilitate Ca^{2+} release. Because mutations/ablation in CS2 result in a similar phenotype as mutations of RyR2 in cardiac muscle (CPVT), it is possible to hypothesise that mutations in CS1 may result in a similar myopathy as mutations in RyR1 in skeletal muscle (*MH and/or CCD*). We tested *in vivo* the sensitivity of CS1-*null* mice to heat-stress and to exposure to halothane. Surprisingly, both treatments were lethal in the majority of CS1 null mice, whereas identical treatments were well-tolerated by WT mice. These crisis were remarkably similar to those described as *fulminant malignant hyperthermia (MH) episodes* in knock-in mice carrying RyR1-MH mutations (Chelu et al. 2006, *Faseb J. 20*:329; Yang et al. 2006, Anesthesiology *105*:1164), suggesting that ablation of CS1 could indeed cause a syndrome similar to MH. To determine if these crisis were associated with functional alteration of skeletal muscle similar to MH muscle, we performed *in vitro* studies of adult EDL muscles and FDB fibers over a temperature range of 25–45°C. Whereas in CS1-*null* specimens, there is a balance between Ca^{2+} release and uptake at low temperatures, above 37°C Ca^{2+} accumulates in the cytosol causing progressive contracture while in *Wt* the balance between uptake and release is maintained for a wider range of temperature. These results may advance our understanding of the molecular mechanisms leading to malignant hyperthermia (MH) in humans and possibly provide an alternative genetic locus for linkage studies.

2688-Plat Calsequestrin Determines Refractoriness Of Calcium Release From Sarcoplasmic Reticulum Of Cardiac Muscle

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Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptor (RyR) channels plays a central role in cardiac excitation contraction coupling. It is not fully understood why SR Ca^{2+} release cannot be elicited by very premature stimuli in intact cardiac tissue. Here, we use calsequestrin null mice (Casq2−/−) and wild-type littermates (Casq2+/+) to test the hypothesis that calsequestrin determines the refractoriness of SR Ca^{2+} release. Casq2−/− hearts and cardiomyocytes display a faster recovery of Ca^{2+} release and depletion as measured using fluorescent dyes sensitive to Ca^{2+} concentrations in cytosol and SR lumen. The kinetics of the luminal Ca^{2+} signal during Ca^{2+} reuptake are slightly faster in Casq2−/− compared with Casq2+/+ hearts, suggesting the possible direct influence of Ca^{2+} buffering by Casq2. We find that SR Ca^{2+} release in response to a premature stimulus (S2) occurs significantly earlier in isolated myocytes and intact hearts lacking calsequestrin. Moreover, when calsequestrin is absent, SR release becomes directly proportional to SR reuptake. As a result, the frequency dependency of SR Ca^{2+} release (negative staircase) was significantly altered. Furthermore, SR Ca^{2+} release alternans occurs at significantly higher pacing frequencies in Casq2−/− (8 Hz) compared with Casq2+/+ hearts (5 Hz, $p<0.01$). Simultaneous measurements of free Ca^{2+} in the SR demonstrate an earlier onset of premature depletion transients. These data demonstrate that calsequestrin determines SR Ca^{2+} release refractoriness by two independent mechanisms:

1. direct regulation of RyR channels and
2. intra SR Ca^{2+} buffering.

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2689-Plat Role of Luminal Ca and Calsequestrin in Controlling Local Ca Release in Cardiac Myocytes

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Recently, we showed that ectopic expression of certain variants of cardiac CASQ2 linked to sudden cardiac death exert either gain of function or loss of function effects on intracellular Ca release by either disrupting CASQ2 polymerization required for high capacity Ca binding or impairing CASQ2 interaction with the RyR channel complex. To gain further insights in the role of CASQ2 in SR Ca signaling, we compared the effects of expression of two such mutants, $CASQ2^{G112+5X}$ and $CASQ2^{R33Q}$, on local cytosolic Ca release events (sparks) and associated SR depletion signals ("blinks") measured simultaneously in rat ventricular myocytes. Overexpression of $CASQ2^{WT}$ increased both the amount of Ca released during a Ca spark and the time required for refilling of individual SR cisternae following local Ca release as compared to control, indicative of enhanced Ca buffering within this structure. At the same time, disrupting CASQ2 polymerization by expressing $CASQ2^{G112+5X}$ resulted in reduced local Ca release and accelerated SR refilling with Ca, consistent with reduced Ca buffering inside SR. However, despite profound differences in SR Ca buffering strengths, local Ca release terminated at the same free luminal [Ca] in the control, $CASQ2^{WT}$ and $CASQ2^{G112+5X}$ groups, suggesting that a decline in [Ca]SR is a signal for RyR2 closure. Importantly, by expressing $CASQ2^{R33Q}$ that impairs interaction of CASQ2 with the RyR2 complex, we were able to alter (lower) the [Ca]SR threshold for Ca release termination. We conclude that CASQ2 aggregated in the SR determines the magnitude and duration of Ca release from each SR cisternae by providing a local source of releasable Ca and by mediating the effects of luminal Ca on the RyR2 channels.

2690-Plat Loss Of Cardiac Triadin Decreases Junctional SR Of Cardiac Muscle And Increases Free Calcium In The SR Lumen

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Triadin binds both to the cardiac ryanodine receptor Ca release channel (RyR2) and to calsequestrin, forming a quaternary complex that also includes junctin. It has been postulated that triadin serves a dual role as anchor for calsequestrin in the junctional SR (jSR), and as a regulator of RyR2 channels. We previously reported that deletion of triadin causes a dramatic reduction of triadin's partner proteins RyR2 (−50%), calsequestrin (−60%) and junctin (−90%) in triadin knock-out mice (KO). Paradoxically, SR Ca content of triadin KO cardiomyocytes is significantly increased, and SR Ca fractional release is significantly reduced. Here we used electron microscopy and measurements of free [Ca] in the SR lumen using MagFura2AM to further probe the role of triadin in cardiac muscle. We find that the overall SR surface area and SR volume of triadin KO hearts are unchanged relative to wild type (WT) hearts (SR vol/vol [%] KO 1.55±0.65 vs WT 1.42±0.58). However, the extent to which the jSR forms feet-bearing junctions with the T tubule is reduced by 50% (jSR extent [A.U.] KO 42±13 vs WT 96±27, $p<0.05$). The decrease in feet bearing junctions is strikingly similar to the reduction in RyR2 protein of triadin KO hearts. Interestingly, free $[Ca]_{SR}$ is significantly increased in KO cardiomyocytes (MagFura2 ratio KO 0.085±0.030 vs 0.062±0.039, n=18 each, $p<0.05$). These data demonstrate that triadin is an important regulator of cardiac Ca release unit structure and function. It remains to be determined whether direct regulation of RyR2 channels by triadin, or the alterations in jSR structure are responsible for the increased luminal $[Ca]_{SR}$.

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Platform BH: Membrane Structure, Rafts, Domains and Phase Separation

2691-Plat Neutron Diffraction Studies of Lipopolysaccharide Bilayers

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Lipopolysaccharides (LPS) are a major class of macromolecules populating the surface of Gram-negative bacteria. They contribute significantly to the bacterium's surface properties and play a crucial role in regulating the permeability of its outer membrane. We report on neutron diffraction studies performed on aligned, self-assembled bilayers of LPS isolated from *Pseudomonas aeruginosa* PAO1. This LPS system comprises of a mixture of rough and smooth A-band and B-band LPS, similar to that naturally found in *P. aeruginosa*. From the construction of one-dimensional scattering length density profiles, we found that water penetrates into the hydrocarbon region up to and including the center of liquid crystalline Na^+-LPS bilayers. This permeability to water also extends to bilayers in the continuous phase transition region and could have far-reaching implications as to how small molecules penetrate the outer membrane of Gram-negative bacteria. Most recently, we have studied the permeability of LPS bilayers to water in the presence of Mg^{2+} and Ca^{2+} cations. We found that, compared to Na^+ and Mg^{2+}, Ca^{2+} causes the bilayers to be less permeable to water.